DNA-DNA hybridization analysis of *Streptococcus thermophilus* plasmids

Key words: *Streptococcus thermophilus*; DNA-DNA hybridization; Plasmid DNA homology

1. SUMMARY

Five of the nine known plasmid DNAs of *Streptococcus thermophilus* ranging from 2.2 kbp to 14.7 kbp in molecular size, were found to share homologous sequences as evidenced by DNA-DNA hybridization analyses. The four other *S. thermophilus* plasmids also lacked homology with one another implying that they may represent unique DNA homology groups. The lack of homology between the *S. thermophilus* probe (pER8, 2.2 kbp) and plasmids of *Lactococcus lactis, L. cremoris* and lactobacilli also implied a fundamental difference in the origins of plasmids of thermophilic and mesophilic lactic acid bacteria.

2. INTRODUCTION

Streptococcus thermophilus is an essential industrial microorganism used in the production of a variety of fermented dairy foods (yoghurt and cheeses). Its primary function is the synthesis of lactic acid from lactose [1]. Other metabolic activities including limited enzyme action on milk proteins [2], and lipids [3], contribute to the development of flavor properties that characterize each food product.

The food-grade nature of *S. thermophilus* has stimulated interest in the genetic engineering development of this organism as a production system for enzymes and bioactive metabolites with potential application in the food processing industries. The discovery of plasmid DNAs [4,5] and restriction endonuclease systems [6] in *S. thermophilus* as well as the application of traditional [7] and the development of new [8] gene transfer techniques represent progress toward the efficient application of recombinant DNA technology to this species. Still to be developed are stable, small, and readily selectable genetic cloning vectors, preferably based on known indigenous plasmids of *S. thermophilus*. To facilitate the achievement of this goal, we initiated studies to identify DNA homology groups among *S. thermophilus* plasmids and to detect sequence homology with plasmid DNAs of mesophilic lactococci and lactobacilli.

3. MATERIALS AND METHODS

3.1. Microbial strains and growth conditions

The 9 strains of *Streptococcus thermophilus*, 10 strains of *Lactococcus lactis*, 2 strains each of *L. cremoris* and *Lactobacillus casei*, and 1 strain each of *L. bulgaricus* and *L. acidophilus* were from our laboratory culture collection. All cultures used in the study were grown at 37°C and stored in a lactose broth medium as previously described [9].

3.2. Plasmid DNA isolation and purification

Plasmid DNAs were isolated from 200 ml broth cultures following overnight growth according to a standard protocol [10]. Purified plasmid preparations were obtained by agarose gel electrophoresis, electroelution, minicolumn adsorption, and cesium chloride density gradient centrifugation [5].

3.3. Biotinylated plasmid DNA probes

Biotinylated probes were developed from the intact *S. thermophilus* plasmids pER8 (2.2 kbp), pER371 (2.7 kbp), pER13 (4.2 kbp), and pER342 (14.7 kbp) or following linearization with an appropriate single-cutting restriction endonuclease [5]. Restriction endonucleases and biotinylated *Hin*dIII fragments of λ DNA were purchased from BRL Life Technologies, Inc. (Gaithersburg, MD). The nick-translation kit and biotin-labeled nucleotides were used as recommended by the manufacturer (Oncor, Inc., Gaithersburg, MD). The biotinylation method was based on the procedure developed by Leary et al. [11].

Biotinylated probes were also prepared from the 0.45 kbp (probe I), 0.75 kbp (probe II), and 1.0 kbp (probe III) fragments of pER8 following double restriction with *Bst*EII and *Hin*dIII.

3.4. DNA-DNA hybridization and detection

DNA sequence homologies were detected by an automated system based on the method of Southern [12], as recommended by the manufacturer (Oncor, Inc.). Intact target plasmid DNAs or their restriction fragments were electrophoresed in 0.7% and 1.0% agarose, respectively, in Tris-borate-EDTA buffer [5]. After depurination and denaturation, DNAs were vacuum transferred to nylon membranes [13] and hybridization with biotinylated probes (12.5 ng/ml) were carried out at 45% formamide concentration in sealed bags for 18 h at 42°C. Post-hybridization washes, filter blocking, and detection of homologous sequences, including streptavidin and alkaline phosphatase treatment, staining with nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate, were performed according to the protocol of the 'Automated Southern Blot System' (Oncor, Inc.).

Table 1

Plasmids of *Streptococcus thermophilus* in DNA-DNA hybridization studies

Plasmid	Size, kbp	Restriction digest	Number of fragments generated	Used as probe (P) or target (T) DNA
pER8	2.20	*Hpa*I	1	P/T
		*Hin*fI	2	T
		*Bst*EII/*Hin*dIII	3	P
pER371	2.70	*Bst*EII	1	P/T
pER341	2.77	*Hae*III	1	P
		*Hin*fI	4	T
pER36	3.70	*Acc*I	1	T
		*Hin*fI	5	T
pER13	4.23	*Bst*EII	1	P/T
pER16	4.46	*Hae*III	1	T
		*Hin*fI	8	T
pER342	9.54	*Acc*I	1	P/T
pER35	11.0	*Pst*I	1	P
		*Hpa*II	6	T
		*Hin*fI	≥14	T
pER372	14.75	*Pst*I	1	T

4. RESULTS

4.1. Biotinylated probes and target DNAs

The plasmids of *S. thermophilus* developed either as biotinylated probes or used as target DNAs in hybridization trials are listed in Table 1. The plasmids pER8, pER36, pER13 and pER35 occur as single entities in various *S. thermophilus* strains, whereas pER341 and pER371 coexist with the larger companion plasmids pER342 and pER372, respectively. Digestion of these plasmids with restriction endonucleases and the construction of seven detailed maps were previously described [5].

In the initial phase of the study, undigested plasmid DNAs (ca. 125 ng) were developed as biotinylated probes or used as targets after agarose gel electrophoresis and vacuum transfer to nylon membranes. However, the strength of hybridization signals (i.e. color band intensity) could be substantially improved by the linearization of each plasmid DNA with an appropriate single-recognition site restriction endonuclease before use as either probe or target. The selection of restriction endonucleases was based on mapping data available on *S. thermophilus* plasmids [5].

4.2. Homology of pER8 with other S. thermophilus plasmids

In Southern hybridization tests, *Hpa*I-digested and biotin-labeled pER8 (2.2 kbp) shown in Fig. 1, probed positively with four larger plasmids found in different *S. thermophilus* strains. Strong hybridization signals were obtained with plasmids pER8 (positive control), pER341, pER36, pER16 (Fig. 1, lanes 1, 3, 4, 6), all predigested with single-recognition site restriction endonucleases, as well as *Hpa*II-digested pER35 (data not shown), indicating the presence of extensive sequence homologies in these plasmids and implying a common ancestral origin. The remaining *S. thermophilus* plasmids, pER371, pER13, pER342, and pER372 failed to show any detectable sequence homology with pER8.

Attempts were made to define areas of pER341 (2.77 kbp), pER36 (3.7 kbp), pER16 (4.46 kbp), and pER35 (11.0 kbp) that harbored the homologous sequences responsible for the strong hybridization signals with biotin-labelled pER8 (2.2 kbp). In these experiments, pER8 was first digested in combination with *Bst*EII and *Hin*dIII, yielding fragment I (0.45 kbp, coordinates 1.05–1.5), fragment II (0.75 kbp, coordinates 0.3–1.05 kbp) and fragment III (1.0 kbp, coordinates 1.5–0.3 kbp), as shown in Fig. 2. The three fragments were subsequently nick-translated, biotinylated and used as probes I, II, and III. These probes were individually hybridized to *Hin*fI digests of pER341 (4 fragments), pER36 (4 fragments), pER16 (8 fragments), and pER35 ($\geq$ 14 fragments), which were resolved by agarose gel electrophoresis (Fig. 3A) Fig. 3B shows that probe I gave strong hybridization signals with the ca. 1.2 kbp *Hin*fI fragment of pER16 (lane 1) and pER341 (lane 3). The probe also strongly hybridized to the ca. 1.3 kbp *Hin*fI fragment present in both pER36 (lane 2) and pER35 (lane 4). Probe I gave only moderate signals with the 0.5-kbp fragment of pER36 (lane 2) and the 0.54-kbp fragment of pER35 (lane 4), and its homology was even less extensive with the 0.7-kbp fragments of pER16 (lane 1), pER36 (lane

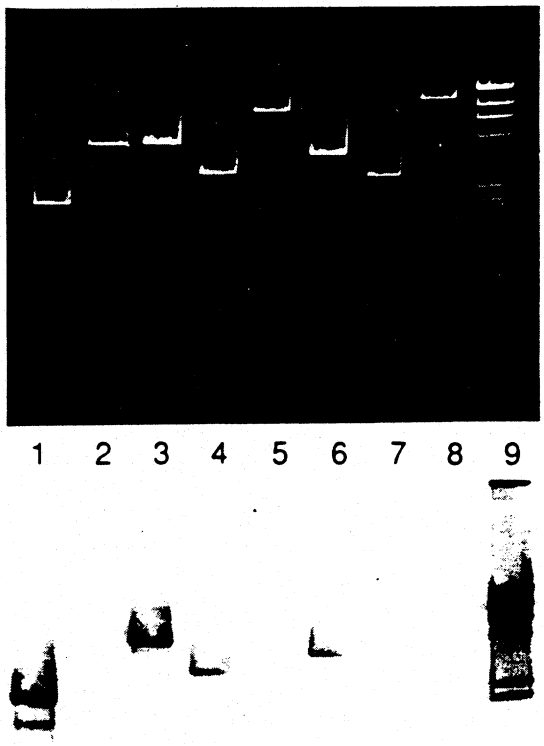

Fig. 1. Hybridization of biotinylated pER8 DNA to plasmids isolated from *S. thermophilus*. Top: agarose gel electrophoresis of restriction digests of plasmids. Bottom: corresponding hybridization of the pER8 probe to a nylon membrane containing linearized plasmid DNAs shown above. Lanes: 1, *Hpa*I digest of pER8 (positive control); 2, pER13 (*Bst*EII); 3, pER16 (*Hae*III); 4, pER341 (*Hae*III); 5, pER342 (*Acc*I); 6, pER36 (*Acc*I); 7, pER371 (*Bst*EII); 8, pER372 (*Pst*I); 9, biotinylated *Hin*dIII fragments of λ DNA.

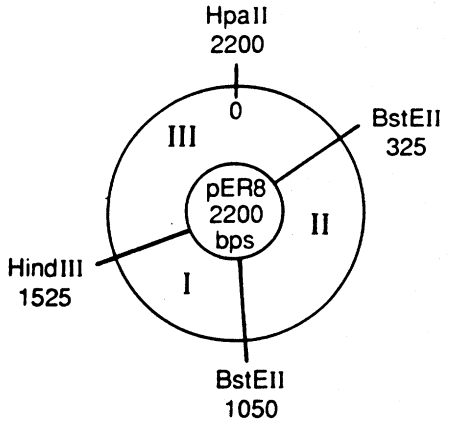

Fig. 2. Restriction enzyme map of pER8 DNA used in the development of probe I (coordinates 1.05–1.5 kbp), probe II (coordinates 0.3–1.05 kbp), and probe III (coordinates 1.5–0.3 kbp), following digestion with *Bst*EII and *Hin*dIII.

2), and pER35 (lane 4) and the 0.56-kbp fragment of pER341 (lane 3).

The intensity of hybridization signals with probe II (0.75 kbp) was strong with the 0.7-kbp *Hin*fI fragment of pER16, pER36, and pER35 (Fig. 3C, lanes 1, 2, 4), as well as 0.56-, 0.54-, and 0.4-kbp fragments of pER341 (Fig. 3C, lane 3).

The hybridization results with Probe III (ca. 1.0 kbp) are shown in Fig. 3D. This probe gave strong signals with the 1.2-kbp fragment of pER16 (lane 1) and the 1.3-kbp fragments of pER36 and pER35 (lanes 2, 4). Probe III hybridized less extensively with other *Hin*fI fragments of pER16 (0.7 kbp), pER36 (0.7 kbp and 0.4 kbp) and pER35 (0.35 kbp) and its signals were also of moderate intensity with the 1.2 kbp and 0.56 kbp fragments of pER341 (lane 3).

Other, weaker signals, obtained with probes I, II, and III that are visible in lanes 1–4 in Fig. 3B–D were assumed to correspond to partially digested forms of the four *S. thermophilus* plasmids and the presence of overlapping sequences in restriction fragments of target DNAs.

4.3. Hybridization with plasmids of lactococci and lactobacilli

*Hpa*I-linearized and biotinylated pER8 was also checked for sequence homology with purified plasmid preparations isolated from twelve strains of lactococci and four strains of lactobacilli. The *S. thermophilus* plasmid failed to hybridize with any of the plasmids which ranged in size from 1.5 to 50 kbp.

4.4. Hybridization with other probes

Biotinylated probes were also developed from *S. thermophilus* plasmids pER371 (2.7 kbp), pER13 (4.23 kbp), and pER342 (9.54 kbp) which

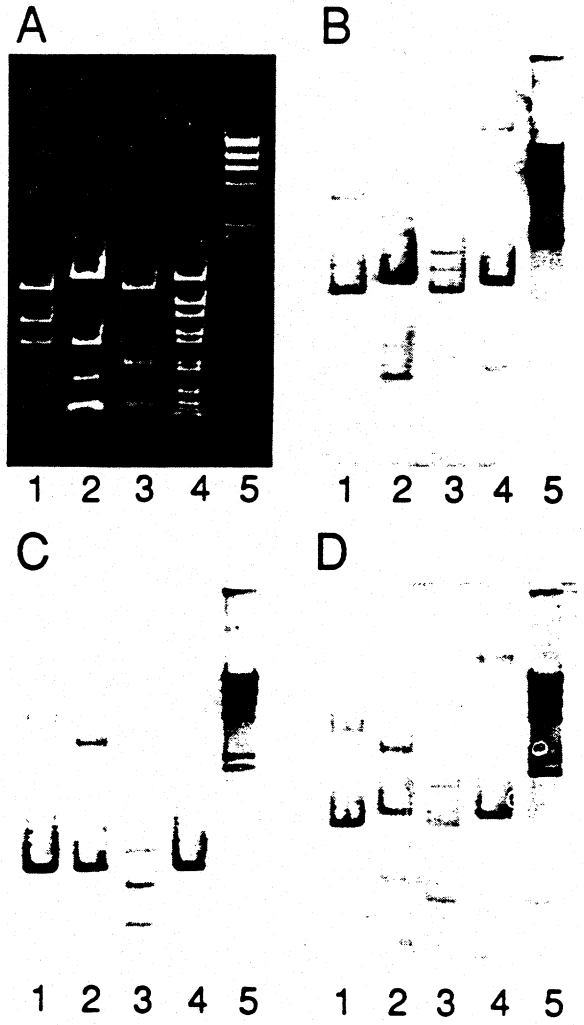

Fig. 3. Hybridization of biotinylated *Hin*fI fragments of pER8 DNA to *Hin*fI fragments of plasmids isolated from *S. thermophilus*. (A) Agarose gel electrophoresis of *Hin*fI fragments. (B–D) corresponding hybridization of probes I, II, and III developed from pER8 DNA to *Hin*fI fragments shown in A. Lanes: 1, pER16; 2, pER36; 3, pER341; 4, pER35; 5, biotinylated *Hin*dIII fragments of λ DNA.

had failed to probe positively with pER8. These probes were used in various combinations with single-restricted plasmid DNAs to test for possible sequence homologies. None of these experiments indicated the presence of shared sequence homologies in these plasmids.

5. DISCUSSION

The results of DNA-DNA hybridization studies indicated the presence of several plasmid homology groups in *S. thermophilus*. The linearized plasmids pER8, pER34 1, pER36, pER16, and pER35 gave strong hybridization signals with the biotinylated pER8 probe. In addition, individual probes developed from restriction endonuclease fragments of pER8 (0.45, 0.75, and 1.0 kbp) strongly hybridized to specific *Hin*fI fragments generated from four plasmid DNAs. These data confirmed that the five plasmids share sequences of homogeneous composition although structurally they are heterogeneous and vary from 2.2 to 11.0 kbp in molecular size [5]. Since the cumulative sums of *Hin*fI fragments of pER341 (ca. 2.3 kbp), pER36 (ca. 2.5 kbp), pER16 (ca. 1.9 kbp), and pER35 (ca. 2.8 kbp) that reacted positively with probes I, II, or III were quite similar to the molecular size of pER8 (2.2 kbp), it was probable that the latter may be identical with or closely related to the common ancestral plasmid from which the other four plasmids evolved. The results also implied that the five plasmids comprising the pER8 homology group may have identical sequences that regulate their replication functions.

Four other plasmids used in the study, pER13, pER371, pER342, and pER372, apparently did not possess homologous sequences with either pER8 or one another. Thus, these plasmids may be viewed as representatives of distinct homology groups. It was also interesting to note that as probes, pER341 and pER371 failed to hybridize to their larger companion plasmids, pER342 and pER372 respectively, indicating the unrelatedness of their evolutionary origins. The results also implied that cloning vectors developed from members of the pER8 plasmid homology group could be introduced efficiently into strains of *S. thermophilus* carrying pER13, pER342, pER371, and/or pER372, without the complications of plasmid incompatibility caused by the presence of homologous components [14].

The failure of biotin-labelled pER8 to hybridize to plasmids isolated from 12 strains of lactococci and four strains of lactobacilli indicated that at least five plasmid DNAs found in *S. thermophilus* (i.e. the pER8 homology group) did not share a common origin with plasmids harbored by mesophilic lactic acid bacteria.

The information generated by the experiments described in this study will be valuable in the characterization of the replication function of *S. thermophilus* plasmids and their development as cloning vectors for the genetic engineering of this important industrial microorganism.

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